

ANTIBIOTIC EFFECTS OF DIMETHYL SULFOXIDE AND
SYNTHETIC POLYPHENOLS ON E. COLI CELLS

Camila Cardenas
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COLUMBUS STATE
UNIVERSITY

ANTIBIOTIC EFFECTS OF DIMETHYL SULFOXIDE AND SYNTHETIC
POLYPHENOLS ON E. COLI CELLS

A THESIS SUBMITTED TO THE

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IN PARTIAL FULFILLMENT OF THE

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BACHELOR OF ARTS

DEPARTMENT OF CHEMISTRY

COLLEGE OF LETTERS AND SCIENCES

BY

CAMILA CARDENAS

Abstract

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There is increasing interest in polyphenols due to their potential medicinal and antibiotic applications. Naturally occurring polyphenols, such as curcumin, have garnered tremendous attention. However, synthetic polyphenols commonly used in biochemistry experiments have not been sufficiently explored. The purpose of this study is to determine the effect of dimethyl sulfoxide on the cellular uptake of synthetic polyphenols. Three synthetic polyphenols (Phenol Red, Congo Red, and Bromophenol Blue) were tested for their antibiotic properties and compared to the antibiotic activity of curcumin. Our results show that phenol red does not interact with the bacterial membrane even in the presence of 30% DMSO, while Bromophenol Blue and Congo Red exhibit weak inhibition of bacterial growth.

Abstract

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I would like to dedicate this thesis to my mother Esperanza Hernandez, for your love and support. I would like to thank Dr. Karl Taylor for the wisdom that he shared with me over my undergraduate career. I want to acknowledge Dr. Karl Taylor for her research assistance during the study. Additionally, I want to thank the Department of Biology for permitting us to use some of their equipment for data collection. Finally, I want to thank the Department of Chemistry at Columbus State University for funding the project and the Columbus State University SPACE grant and the Honors College for additional funding.

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I. Introduction

The last twenty years have seen a massive shift by the pharmaceutical industry away from traditional synthetic small molecule drugs and towards a broad class of molecules collectively called biologics. Biologics are compounds that are synthesized in part or in whole from living systems (1). While biologics can be any range of biological compounds, including carbohydrates, nucleic acids, or even combinations of these, the class that has garnered the most interest and investment from the pharmaceutical industry are proteins and peptides. A recent study indicated that well over 200 peptides and proteins have been approved by the Food and Drug Administration for clinical use. In addition to these natural sequences, another 380 approved drug variants were reported (2). Despite the success of these drugs, the development of many potential protein and peptide-based drug candidates have been hindered by inadequate analytical methods. Unfortunately, many proteins and peptides of pharmaceutical interest, either as drug candidates or drug targets, exhibit a strong tendency to stick together into inactive and sometimes insoluble aggregates. This aggregation can reduce biosynthetic yields of active monomers and slow down or completely hinder research efforts.

A number of new techniques and methodologies have been developed in order to improve the yield of these aggregation prone proteins and peptides. However, the results usually do not extend beyond the molecule of interest and researchers must optimize production conditions for each new protein and peptide. Often even changing a single amino acid in a protein can require starting the optimization process anew.

A. Production of Aggregation-Prone Proteins

The last twenty years have seen a massive shift by the pharmaceutical industry away from traditional synthetic small molecule drugs and towards a broad class of molecules collectively called biologics. Biologics are compounds that are synthesized in part or in whole from living systems (1). While, biologics can be any range of biological compounds, including carbohydrate, nucleic acids, or even combinations of these, the class that has garnered the most interest are proteins and peptides. A recent study indicated that well over 200 peptides and proteins have been approved by the Food and Drug Administration for clinical use. In addition to these natural sequences, another 380 approved drug variants were reported (2). Despite the success of these drugs, the development of many potential protein and peptide-based drug candidates have been hindered by inadequate biosynthetic methods. Unfortunately, many proteins and peptides of pharmaceutical interest, either as drug candidates or drug targets, exhibit a strong tendency to stick together into inactive and sometimes insoluble aggregates. This aggregation can reduce biosynthetic yields of active monomers and slow down or completely hinder research efforts.

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While many studies have focused on using polyphenols to prevent aggregation *in vitro* and in eukaryotic cells, their use to prevent aggregation in bacteria used for protein

B. Anti-aggregation properties of Polyphenols

Polyphenols are molecules that contain two or more phenol functional groups. Phenols are cyclic aromatic compounds with a hydroxyl (OH) group attached to one of the rings six carbon atoms. There are more than 8,000 known natural and synthetic polyphenols. Natural polyphenols are a class of phytochemicals, which are compounds found abundantly in plants. There is high interest in the *in vitro* applications of polyphenols due to their anti-inflammatory, antibiotic, anti-cancer, and antioxidant properties (3, 5, 6,).

Research has shown that some polyphenols, such as curcumin (Figure 1), are effective at inhibiting the formation of higher order protein aggregates *in vitro* (4). Some natural polyphenols bind to misfolded amyloid beta (A β) proteins, a major hallmark of Alzheimer's disease, and prevent their aggregation into A β -fibrils by interfering with the stacking of the aromatic side chains therefore, disrupting the amyloid structure (7, 17, 18).

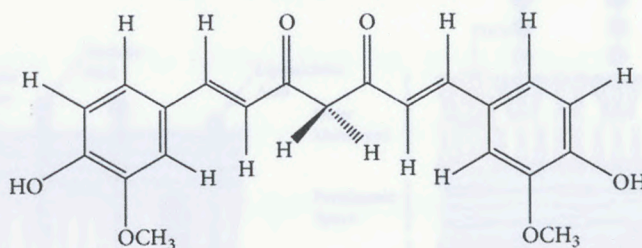


Figure 1: Structure of the naturally occurring polyphenol curcumin

C. Polyphenol use in Prokaryotic cells

While many studies have focused on using polyphenols to prevent aggregation *in vitro* and in eukaryotic cells, their use to prevent aggregation in bacteria used for protein

expression has not been explored. Two of the major hurdles to the use of polyphenols as aggregation inhibitors in bacteria is the antibiotic activity of many polyphenols and their apparent inability to cross the bacterial cell membrane.

In eukaryotic cells, the cell membrane is the outermost layer and it separates the cellular contents from the external environment. Meanwhile, the outermost layer of prokaryotic cells is the cell wall which surrounds the cell membrane. The cell membrane has the same function for both, eukaryotic and prokaryotic cells. Both, bacteria and animal cells contain outer membrane porins that are transmembrane proteins which are usually known to form channels that enable the passage of hydrophilic molecules such like phenol, chlorophenol and antibiotics (14). If the bacterial porins contain a mutation, the result would be antibiotic resistance by blocking the antibiotic entrance to the prokaryotic cell. However, their permeability to a given molecule can be very different. While, many polyphenols have been shown to easily diffuse through eukaryotic membranes, some of those same molecules are potent antibiotics.

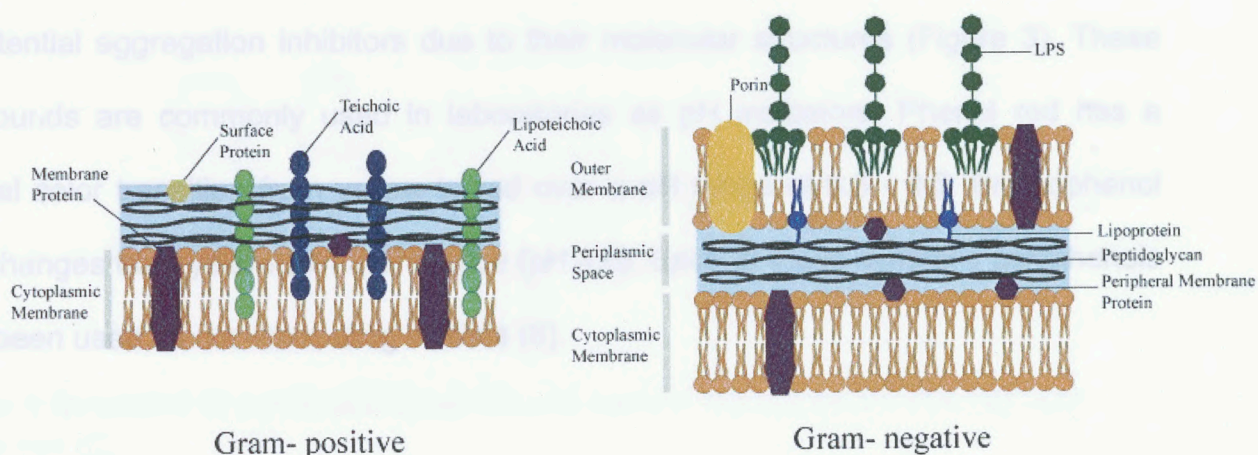


Figure 2: Gram-negative vs. Gram-positive bacteria (19)

The most common prokaryote used for protein expression of biologics is the gram-negative bacteria *Escherichia coli* (*E. coli*). Gram-negative bacteria often have a dual membrane system which consists of two plasma membranes on either side of a cell wall (Figure 2). This extra layer of protection forces larger molecules to cross the membrane by facilitated diffusion (13). Vaara explains, the additional layer “provides the cell with an effective permeability barrier against antibiotics but is itself a target for antibacterial agents” (15). This means that for a polyphenol to be useful as an aggregation inhibitor in *E. coli*, it must satisfy three criteria: it must cross the cell wall, it must not exhibit antibiotic activity, and it must retain inhibitive activity inside the cell.

D. Synthetic polyphenols: Phenol red, Congo red, and Bromophenol blue

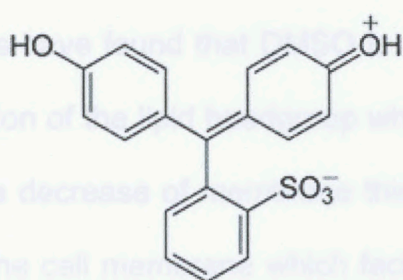
While natural polyphenols have garnered much attention for potential medicinal properties, many common synthetic polyphenols have been overlooked in the search for novel anti-aggregation agents. However, phenolsulfonphthalein (phenol red) and 3',3'',5',5''-Tetrabromophenolsulfonephthalein (bromophenol blue) have been identified as potential aggregation inhibitors due to their molecular structures (Figure 3). These compounds are commonly used in laboratories as pH indicators. Phenol red has a gradual color transition from yellow to red over a pH range of 6.8 - 8.2. Bromophenol blue changes from yellow (pH 3.0) to blue (pH 4.6). Each of these synthetic polyphenols have been used as non-toxic drug models (8).

Figure 3 Structures of synthetic dyes, Phenol red (A), Bromophenol blue (B), and Congo red (C).

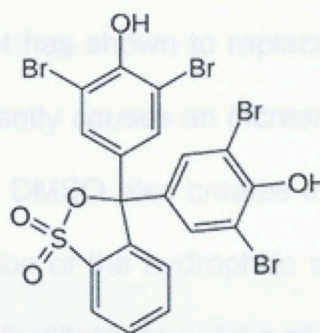
E. Congo red: An azo dye

F. Dimethyl sulfoxide

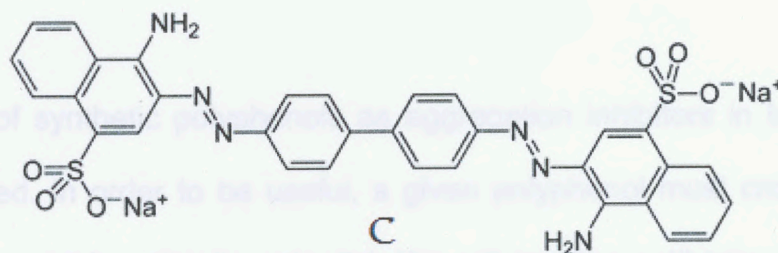
Congo red is also used as a pH indicator and transitions from blue to red (pH 3.0 - 5.3). Congo red is an organic compound that contains two azo groups and aromatic rings but lacks aromatic phenolic rings. While it is not a polyphenol, it has been shown to inhibit aggregation in a manner similar to polyphenols and readily adsorbs to gram-negative bacteria. Therefore, it is commonly included in anti-aggregation studies alongside polyphenol compounds. However, the use of Congo red has been limited in experimental settings due to its carcinogenic properties (9).



A



B



C

Figure 3 Structure of synthetic dyes. Phenol red (A), Bromophenol blue (B), and Congo red (C).

F. Dimethyl sulfoxide

It has been shown that bacterial uptake of antibiotics can be increased in the presence of the chelator ethylenediaminetetraacetic acid (EDTA) or the solvent dimethyl sulfoxide (DMSO). DMSO is a polar aprotic solvent that has amphiphilic properties since it is composed of two nonpolar methyl groups and sulfinyl which is a water-soluble group. Coincidentally, due to this property, DMSO is miscible in multiple solvents, including water. Studies have shown that DMSO is an excellent solvent to dissolve stimulants and drugs as it increases the membrane permeability therefore, assisting with facilitated diffusion across biological membranes (10).

Studies have found that DMSO is a solvent that has shown to replace water in the "inner region of the lipid headgroup which consequently causes an increase of area per lipid and a decrease of membrane thickness" (11). DMSO also creates transient water pores in the cell membrane which facilitates diffusion of the hydrophilic solvent across the plasma membrane (12). Therefore, DMSO may facilitate the uptake of polyphenols.

G. Proposal

The use of synthetic polyphenols as aggregation inhibitors in bacteria remains largely unexplored. In order to be useful, a given polyphenol must cross the bacterial cell wall and not exhibit antibiotic potential. We will test the antibiotic properties of the three synthetic polyphenols. Additionally, initial studies in our lab have shown that synthetic polyphenols do not readily cross the cell membrane. Therefore, the effect of dimethyl sulfoxide on the cellular uptake of Congo red, bromophenol blue, and phenol red which will be explored.

II. Materials and Methods

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A. Determination of isosbestic point

C. Antibiotic Activity of Polychandris

Changing cell culture conditions and workup in buffers of various pH values can have drastic effects on the molar absorptivity values of compounds. Therefore, it is necessary to measure absorbance values at the isosbestic point. The isosbestic point is the wavelength that a compound has a constant molar absorptivity regardless of pH. The isosbestic point was found by diluting the stock solution of each polyphenol in phosphate buffered saline (PBS) solutions at various pH values. The absorbance spectrums of the resulting solutions were measured using a UV-Vis spectrophotometer. The spectrum was scanned between 400 nm to 600 nm, in 1 nm increments. The results were analyzed using Microsoft® Excel®. The wavelength at which the molar absorptivity was unchanged, the isosbestic point, was used for all subsequent measurements.

B. Effect of DMSO concentration on cell growth

DMSO (99%, SigmaAldrich) was diluted with PBS (pH 7.4) to give a 10%, 8%, 6%, 5%, and 3% solutions. A starter culture of *E. coli* (BL21) cells were incubated overnight at . An aliquot of the starter culture was then incubated in one of the DMSO/PBS solutions for 30 minutes. The treated cells were then spread on Luria broth (LB) agar plates and incubated overnight at . The following morning the plates were compared to determine the effects of DMSO on cell growth.

III. Results and Discussion

C. Antibiotic Activity of Polyphenols

In order to determine the feasibility of using the synthetic polyphenols, they were screened for antibiotic activity. A starter culture of *E. coli* (BL21) cells were incubated overnight at . The following morning, the starter culture was used to inoculate a 250 mL culture. Once the cells reached the exponential growth phase ($OD_{600} > 0.5$), the cells were diluted and spread on LB agar plates. Three diffusion disks treated with 20 μ L the respective polyphenols (2 mM) and controls were added to the plates. Plates were incubated overnight at 37 and examined the following morning for antibiotic activity.

A. DMSO Effects

III. Results and Discussion

Previous publications have attempted to show the antimicrobial activity of DMSO against *E. coli* by increasing the DMSO levels and testing the growth inhibition. However, these publications did not address the changes that result from increasing cell density at concentrations lower than 15% DMSO [16]. This research tested the effects of 10%, 8%, and 6% DMSO solutions. The high DMSO concentrations (10% and 8%) showed no cellular growth on the LB plates, while the 6% DMSO showed very little growth.

For this reason, the DMSO concentration was lowered and tested at 5%, 3%, and 1%. Each sample produced an overgrown plate after overnight incubation at 37°C on LB/Ampicillin plates (Figure 4). This confirmed that these concentrations of DMSO do not result in cell death. However, due to variability in the results at 5%, all subsequent experiment used 3% DMSO.



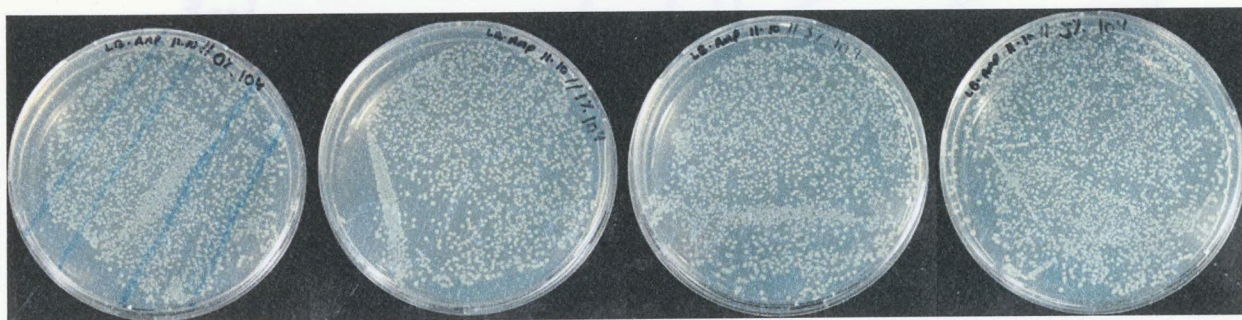
B. Isobestic Point of Synthetic Polyphenols

The isobestic point refers to the wavelength at which the absorbance by a mixed solution remains unchanged as the equilibrium of the two component changes.

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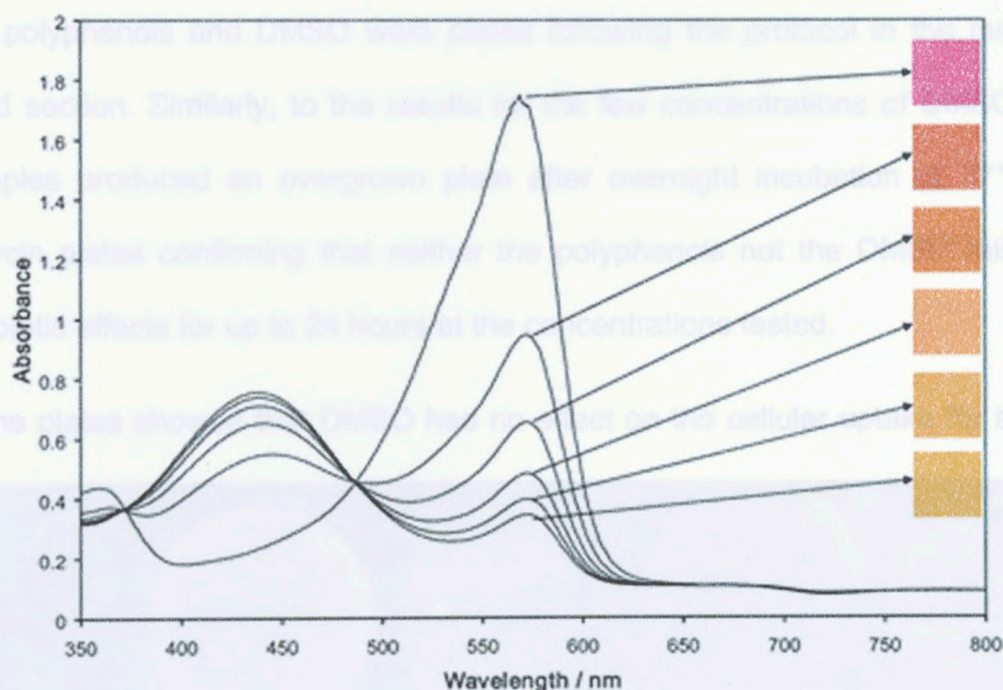
B. Isosbestic Point of Synthetic Polyphenols

C. Antibiotic Effects of Synthetic Polyphenols

The isosbestic point refers to the wavelength at which the absorbance by a mixed solution remains unchanged as the equilibrium of the two component changes.

This means, that at the specific isosbestic point, the absorbance of the dye would not change due to a change in color at different pH. Phenol red, Bromophenol blue, and Congo red are used as indicators due to their ability to change color as a result of varying pH. Since the research intends to use specific absorbance-based concentration measurements, the isosbestic point for each was found.

After repeated measurements, Griffin (12) found the isosbestic point of phenol red to be 478 nm, as shown in Figure 5. After throughout research, the reported isosbestic point for Curcumin, Bromophenol blue, and Congo red were 375 nm, 490 nm, and 541 nm, respectively.



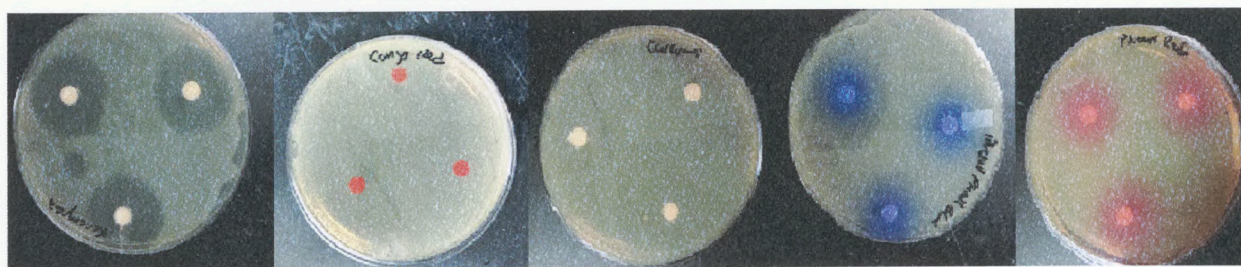
C. Antibiotic Effects of Synthetic Polyphenols

Although polyphenols compounds have been found to prevent aggregation in eukaryotic cells, many have documented antibiotic effects. If any of the polyphenols used had any antibiotic effect, it would limit the effectiveness in bacterial cells for protein expression. For this reason, all polyphenols were tested at different concentrations by growing *E. coli* (BL21) cells in the LB media.

Once it was clear that none of the polyphenols produced cell death, the competent cells were then treated with 2 mM of each polyphenol. At the same time, from the results obtained at low DMSO concentrations, cells were treated at 3% DMSO/PBS solution which was considered to be appropriate for colony forming units.

The polyphenols and DMSO were plated following the protocol in the material and method section. Similarly, to the results for the low concentrations of DMSO, the plated samples produced an overgrown plate after overnight incubation at 37°C on LB/Kanamycin plates confirming that neither the polyphenols not the DMSO failed to exhibit antibiotic effects for up to 24 hours at the concentrations tested.

All the plates showed that DMSO had no effect on the cellular uptake for any of



the respective polyphenols. At the same time, none of the polyphenols affected the bacteria cell growth and neither of them went inside the cell. However, each of the plates showed different effects on the blank paper disks, as shown in Figure 6.

Since kanamycin is an antibiotic, it was our positive control. And so, as expected, it showed cellular apoptosis. Meanwhile, Congo red solution did not disperse on the plate therefore, resulting in no contact with bacteria. On the contrary, the solutions for curcumin, phenol red, and bromophenol blue disperse on the plate over time.

IV. Conclusion and Future Work

As it was previously stated, the study was conducted to determine the effect of dimethyl sulfoxide on the cellular uptake of the three synthetic polyphenols: Congo red, Bromophenol blue, and Phenol red. These dyes were used due to previous research suggesting the antibiotic properties of the polyphenols. Since synthetic polyphenols have been poorly investigated, we completed experimentation to determine their antibiotic properties.

None of the synthetic polyphenols were able to permeate the cell membrane of *E. coli* without DMSO, as the chelator. The study proved that Phenol red was absorbed when incubated in neat DMSO. However, when DMSO was diluted at different concentrations, none of the polyphenols were absorbed. Similarly, the results showed that a concentration of DMSO higher than 8% prevented the *E. coli* cells to properly grow.

Future research is needed to verify the polyphenols and DMSO cellular uptake. Even though the results confirm *E. coli* growth, it was not possible to prove if the resulted poor uptake was due to the polyphenols inability to penetrate the cell membrane or if they did were able to go inside the cell, but no effect on the negative gram bacteria. Additionally, the results proved that neither Phenol red, Bromophenol blue, and Congo red have antibiotic effects, therefore they can be used as plausible candidates for future research to prevent aggregation.

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By

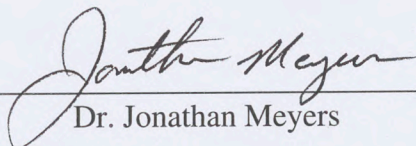
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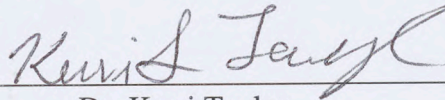
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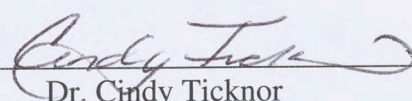
HONORS COLLEGE

In Partial Fulfillment of the Requirements
for Honors in the Degree of

BACHELOR OF ARTS
CHEMISTRY
COLLEGE OF LETTERS & SCIENCES

Thesis Advisor  Date May 15, 2018
Dr. Jonathan Meyers

Committee Member  Date May 15, 2018
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Honors College Dean  Date 5/15/2018
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